Plant Signaling & Behavior 6:3, 422-425; March 2011; © 2011 Landes Bioscience

The N termini of Brassica and tung omega-3 fatty acid desaturases mediate proteasome-dependent protein degradation in plant cells

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Key words: endoplasmic reticulum, fatty acid desaturase, green and red fluorescent proteins, proteasome, protein degradation

Submitted: 12/16/10

Accepted: 12/16/10

DOI: 10.4161/psb.6.3.14522

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Addendum to: O'Quin JB, Bourassa L, Zhang D, Shockey JM, Gidda SK, Fosnot S, et al. Temperature-sensitive, post-translational regulation of plant omega-3 fatty acid desaturases is mediated by the ER-associated degradation pathway. J Biol Chem 2010; 285:21781–96; PMID: 20452984; DOI: 10.1074/jbc.M110.135236.

The regulation of fatty acid desaturase activity in plants is important for determining the polyunsaturated fatty acid content of cellular membranes, which is often rapidly adjusted in plant cells in response to temperature change. Recent studies have demonstrated that the endoplasmic reticulum (ER)-localized omega-3 fatty acid desaturases (Fad3s) are regulated extensively at the post-transcriptional level by both temperature-dependent changes in translational efficiency, as well as modulation of protein half-life. While the N-terminal sequences of Fad3 proteins were shown to contain information that mediates their rapid, proteasome-dependent protein turnover in both plant and yeast cells, it is currently unknown whether these sequences alone are sufficient to direct protein degradation. In this report, we fused the N-terminal sequences of two different Fad3 proteins to an ER-localized fluorescent protein reporter, consisting of the green fluorescent protein and the ER integral membrane protein cytochrome b_5, and then measured (via microscopy) the degradation of the resulting fusion proteins in plant suspension-cultured cells relative to a second, co-expressed fluorescent reporter protein. Overall, the results demonstrate that the N-termini of both Fad3 proteins are sufficient for conferring rapid, proteasome-dependent degradation to an ER-bound marker protein.

Plants are sessile organisms that must rapidly adapt to changes in their environment in order to survive. For instance, one of a plant's main physiological adjustments to cooler temperatures is a marked increase in the polyunsaturated fatty acid (PUFA) content of its cellular membranes, which is thought to help maintain the proper fluidity of membranes and thus, support overall cell function.[1-3] PUFAs are synthesized by a variety of fatty acid desaturase enzymes including the omega-3 desaturases, which are located in either the endoplasmic reticulum (ER) (Fad3) or the chloroplasts (Fad7 and Fad8).[4] The Fad3 proteins are typically short-lived, and their steady-state amount is modulated by temperature through both changes in mRNA translational efficiency as well as alterations in protein half-life.[5,6] Recently, we demonstrated that the half-life of Fad3 proteins is regulated by a combination of cis-acting degradation signals present in their N-terminal regions and proteasomal degradation,[6] but it was not clear whether the N-terminal sequences alone were sufficient for these functions. Here we extend these studies by showing that the N-terminal sequences of two different Fad3 proteins are indeed sufficient to confer rapid, proteasomal-dependent regulation to an ER-localized reporter protein.

The N Termini of Brassica and Tung Fad3 Confer Rapid, Proteasomal-Dependent Degradation to GFP-Cb5

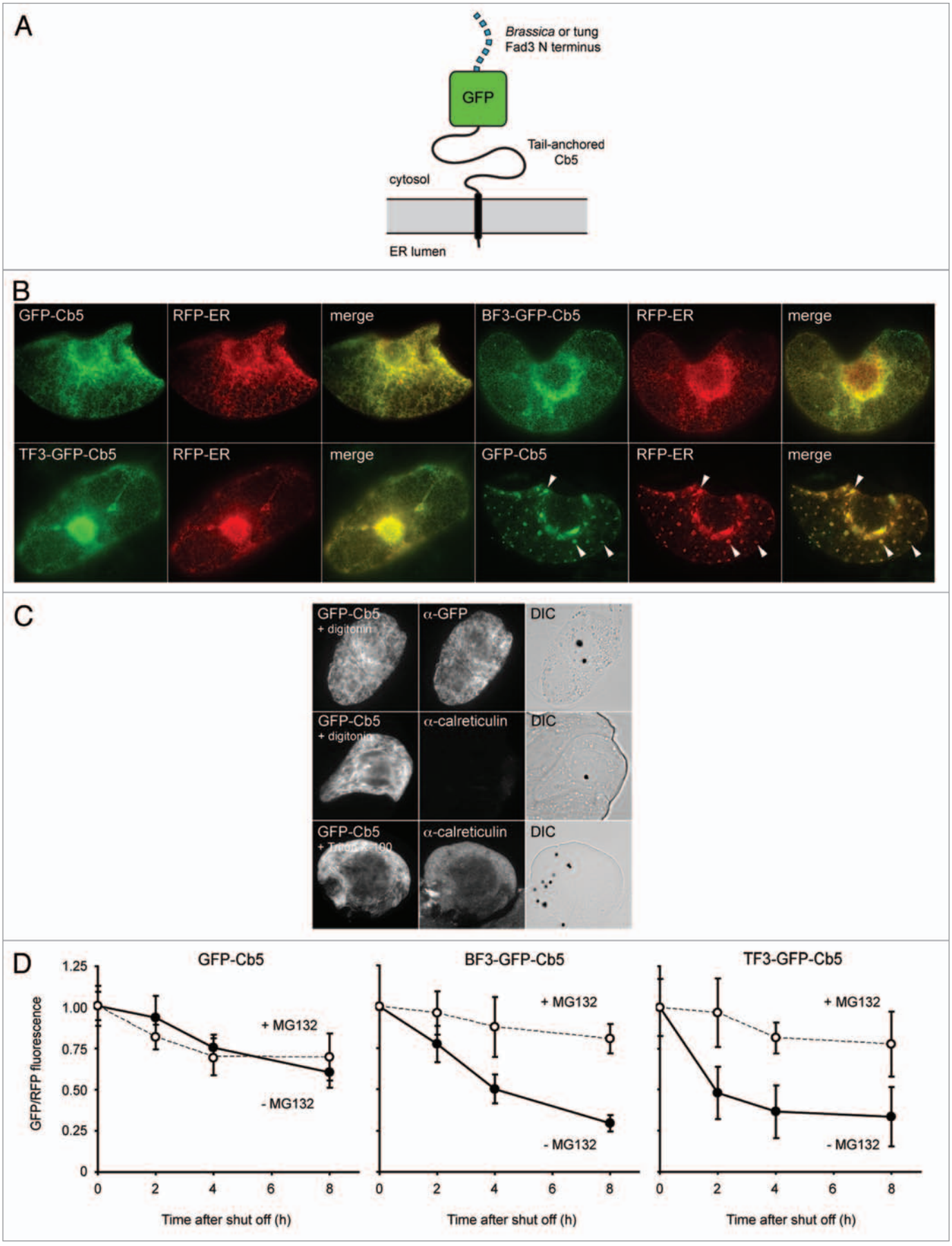
To determine whether the N-terminal sequences of Fad3 proteins were sufficient to confer a short half-life, recombinant DNA techniques were used to fuse the first ~60 amino acid residues

(i.e., the soluble region just prior to the first predicted transmembrane domain) of *Brassica napus* (BF3) or tung (*Vernicia fordii*) (TF3) Fad3 to the N terminus of GFP-Cb5, a well-characterized ER membrane-targeted fusion protein consisting of the green fluorescent protein (GFP) linked to the C-terminal (tail)-anchored ($N_{cytosol}$ - $C_{ER\ lumen}$) ER integral membrane protein cytochrome b_5 (Cb5).[7,8] In doing so, the Fad3 N-terminal sequences in BF3-GFP-Cb5 and TF3-GFP-Cb5 were orientated towards the cytosol, consistent with their orientation in native (full-length) BF3 and TF3 proteins.[9] A cartoon depicting the structure of the Fad3-GFP-Cb5 fusion proteins, as well as their expected $N_{cytosol}$ - $C_{ER\ lumen}$ topology is shown in **Figure 1A**. To measure the degradation rates of the Fad3-GFP-Cb5 fusion proteins in plant cells, we utilized a novel dual fluorescent protein reporter system, as previously described in reference 6. Briefly, tobacco [Bright Yellow-2 (BY-2)] suspension-cultured cells[10,11] were transiently-transformed (via biolistic bombardment) with a double-gene plasmid encoding either GFP-Cb5, BF3-GFP-Cb5 or TF3-GFP-Cb5 and RFP-ER, an ER lumenal-targeted red fluorescent protein (RFP),[6] which serves as an internal control to normalize fluorescence values that may change due to variation in expression levels, plasmid copy number, etc., following biolistic bombardment. The degradation rates of each pair of co-expressed proteins (e.g., GFP-Cb5 and RFP-ER) were then calculated by measuring (via microscopy) the GFP/RFP fluorescence ratio in multiple, individually-transformed cells following inhibition of protein translation with cycloheximide.

Figure 1B and C demonstrate that each of the GFP-Cb5 fusion proteins co-localized exclusively with the RFP-ER marker protein in the ER of representative transiently-transformed BY-2 cells, as expected,[7,8] and adopted the proper topology in ER membranes, whereby their N termini were oriented towards the cytosol (data not shown for BF3-GFP-Cb5 and TF3-GFP-Cb5). Notably, after extended time periods (e.g. >10 h) following biolistic bombardment and/or in transformed cells exhibiting bright fluorescence due to high levels of ectopic (gene) expression, the morphology of the ER was often altered. That is, the ER in these cells exhibited a distinct globular fluorescence pattern (**Fig. 1B** and bottom right panels) that was conspicuously different from the reticular pattern normally observed at earlier time periods and/or cells exhibiting low levels of fluorescence (i.e. ectopic gene expression). These globular structures were presumed to be karmalle, which are stacks of ER cisternae and are often induced by Cb_5 when overexpressed in eukaryotic cells.[7,12,13] While karmalle do not induce the unfolded protein response,[13] any BY-2 cells exhibiting these types of structures were discarded from our analysis of Fad3-GFP-Cb5 protein half-life.

Investigation of protein degradation rates for the various fusion constructs revealed that GFP-Cb5 was moderately stable in plant cells, and notably, the degradation rate was not appreciably affected by inclusion of the proteasomal inhibitor MG132 (**Fig. 1D** and left graph). Addition of either the BF3 or TF3 N-terminal sequence to GFP-Cb5, however, resulted in enhanced protein degradation that was significantly inhibited by inclusion of MG132 (**Fig. 1D** and middle and right graphs), indicating the Fad3 N termini contain cis-acting signals that

Figure 1 (See next page). The N-terminal sequences of *Brassica napus* and tung Fad3 proteins confer rapid, proteasomal-dependent degradation to an ER-localized reporter protein expressed in plant cells. (A) Cartoon model of the Fad3-GFP-Cb5 fusion proteins orientated in an $N_{cytosol}$ - $C_{ER\ lumen}$ manner in the ER membrane. As shown, the fusion proteins consist of three domains: (1) the C-terminal (tail)-anchored ER membrane protein, Cb5 (specifically, Cb5 isoform A from tung),[7] including its short lumenal-facing C-terminal region, single C-terminal TMD (colored black) and cytosolic-facing N-terminal region; (2) monomeric GFP[15] (colored green); and (3) the N-terminal, cytosolic-facing soluble domain of Brassica (amino acid residues 1–59) or tung (residues 1–65) Fad3 (BF3 or TF3; colored blue and stippled). (B) Targeting and subcellular properties of various GFP fusion proteins transiently expressed in tobacco BY-2 suspension-cultured cells. Representative epifluorescence images of living BY-2 cells following biolistic bombardment (~8 h post-bombardment) with dual gene expression binary vectors (based on pRCS2[16]) encoding either GFP-Cb5, BF3-GFP-Cb5 or TF3-GFP-Cb5 (driven by the cauliflower mosaic virus 35S promoter) and the ER lumenal marker protein RFP-ER (driven by the nopaline synthase promoter), consisting of RFP fused to an N-terminal signal sequence and C-terminal (-HDEL) ER retrieval signal. Complete details on the construction of these (three) binary vectors are available upon request. The yellow color in the corresponding merged images indicates co-localization of the co-expressed proteins at the ER. Also shown (bottom row at the right) are representative images of a GFP-Cb5 and RFP-ER co-transformed cell, wherein overexpression (~20 h post-bombardment) of GFP-Cb5 has led to the formation of large, globular fluorescent ER structures (indicated with arrowheads), which, based on the appearance of similar Cb5-induced ER structures in other published studies,[7,12,13] were presumed to be karmellae. (C) Topology mapping of the GFP-Cb5 reporter protein in ER membranes. Representative immuno-epifluorescence images of BY-2 cells transformed with GFP-Cb5 alone, fixed with formaldehyde, and differentially permeabilized (as indicated by the labeling in the left-hand parts of each row) with either digitonin (which permeabilizes only the plasma membrane) or Triton X-100 (which permeabilizes all cellular membranes).[17] Permeabilized cells were then incubated with antibodies raised against (as indicated) either GFP or endogenous calreticulin (a protein located in the ER lumen[17]), along with the appropriate dye-conjugated secondary antibodies. Also shown (in the left-hand part of each row) is the autofluorescence attributable to expressed GFP-Cb5. Note that GFP-Cb5, but not endogenous calreticulin in the ER lumen, was immunodetected in digitonin-permeabilized cells (top and middle rows), whereas endogenous calreticulin was immunodetected in Triton X-100-permeabilized cells (bottom row), as expected.[17] This confirms that the GFP-Cb5 protein adopts an $N_{cytosol}$ - $C_{ER\ lumen}$ orientation. Similar results were obtained for Fad3-GFP-Cb5 fusion proteins (data not shown). (D) Graphs illustrating the degradation of GFP-Cb5 (left graph), BF3-GFP-Cb5 (middle graph) and TF3-GFP-Cb5 (right graph) in BY-2 cells. Based on the procedures described in O'Quinn et al.[6] cells were biolistically bombarded with dual gene expression binary vectors [as above in (A)] encoding either GFP-Cb5, BF3-GFP-Cb5, TF3-GFP-Cb5 and RFP-ER, which served as an internal (fluorescence) control. Following bombardment, cells were incubated for ~3 h to allow for protein expression and intracellular (ER) targeting and then cycloheximide (100 μm) was added (at t = 0 h) to block new protein synthesis, along with (or without) the proteasome inhibitor MG132 (100 μm). Aliquots of cells were then collected at t = 0, 2, 4 and 8 h, fixed, and viewed via epifluorescence microscopy with identical image acquisition settings. Fluorescence intensities of GFP and RFP in selected co-transformed cells ($n \geq 10$) (i.e. cells other than those exhibiting high levels of ectopic [gene] expression and/or containing karmellae) were calculated and plotted as normalized GFP/RFP fluorescence ratios (±ca. standard deviations) according to O'Quinn et al.[6] The results shown in (A–C) are representative of at least three independent experiments.


A
Brassica or tung
Fad3 N terminus
GFP
Tail-anchored
Cb5
cytosol
ER lumen
B
GFP-Cb5
RFP-ER
merge
BF3-GFP-Cb5
RFP-ER
merge
TF3-GFP-Cb5
RFP-ER
merge
GFP-Cb5
RFP-ER
merge
C
GFP-Cb5
+ digitonin
α-GFP
DIC
GFP-Cb5
+ digitonin
α-calreticulin
DIC
GFP-Cb5
α-calreticulin
DIC
D
GFP-Cb5
BF3-GFP-Cb5
TF3-GFP-Cb5
GFP/RFP fluorescence
1.25
1.00
0.75
0.50
0.25
0
+ MG132
- MG132
0
2
4
6
8
Time after shut off (h)

confer rapid, proteasome-dependent protein degradation.

Implications for Understanding Lipid Homeostasis in Eukaryotic Cells and Cold Temperature Response in Plants

The demonstration that the N termini of Fad3 proteins are sufficient to confer protein degradation in plant cells (**Fig. 1D**) confirms and extends our previous observations obtained with full-length Fad3 proteins expressed in either yeast or plant cells.[6] Interestingly, the N terminus of mammalian stearoyl-CoA desaturase has been shown also to be sufficient for conferring rapid protein degradation to a Cb5-GFP reporter protein,[14] suggesting that maintenance of short protein half-life is an important feature of fatty acid desaturase protein regulation, in general. In plants, for instance, having a shorter half-life likely allows for tighter coupling between changes in gene expression, mRNA translation efficiency, and provides an opportunity to modulate steady-state protein amount by altering the rate of protein degradation. This form of regulation would allow for the fine-tuning of fatty acid desaturase activity in response to various environmental changes. Furthermore, in our previous studies, we demonstrated that the Fad3 proteins were degraded in a proteasome-dependent manner in both plant and yeast cells, and we utilized yeast as a model system to identify specific components of the ER associated degradation (ERAD) pathway that were involved in Fad3 degradation.[6] With the development of the Fad3-GFP-Cb5 reporter constructs described here, we now have in hand a robust set of tools to begin to investigate the possible conserved role of ERAD in the regulation and degradation of Fads in plant cells.

Acknowledgements

This work was supported by the United States Department of Agriculture (J.M.D. and J.M.S.), a grant from the Natural Sciences and Engineering Research Council of Canada (to R.T.M.), and a University of Guelph Research Chair (to R.T.M.).

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